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Prostate Cancer (PCa) Cells

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## INTRODUCTION

Apoptosis is a complex biological process encompassing several sequential molecular stages: initial trigger, signal transduction, apoptotic execution, and eventual cell demise. To date, the apoptotic signaling pathways initiated by most of the death triggers remain poorly characterized. Two relatively well-delineated apoptotic signaling pathways involve death receptors (e.g., TNFR1, Fas, and DR3) and cytochrome c (1, 2). In the first, engagement of cell surface death receptors by the ligands leads to the recruitment of the death-domain containing adaptor proteins, and the proximal initiating caspases, resulting in the activation of distal executioner caspases (3). In the second, various inducers including certain chemotherapeutic drugs somehow affect the mitochondria such that holocytochrome c is released into the cytosol. The cytosolic cytochrome c then binds to and activates Apaf-1, which then activates the initiating caspase, i.e., caspase-9, which in turn activates caspase-3 (2).

Most PCa have evolved various mechanisms to subvert apoptotic program and are also resistant to apoptosis induction by most conventional chemotherapeutic drugs. In both cases, we know little about the underlying molecular mechanisms. Attenuated apoptotic response and extended cell survival seem to be closely associated with the initiation, progression and metastasis of Pca, as well as with their resistance to drugs (4-7). Our novel apoptotic model (MAP, Mitochondria-dependent apoptotic pathway) may partially explain the long-term survivability and drug resistance in PCa cells (8-10). My main objective in the proposed grant is to elucidate the following two specific aims:

- 1) **To determine whether increased cytochrome c translocation to mitochondria during MAP induction is required for apoptosis to occur, and**
- 2) **To determine how mitochondrial procaspase-3 is activated in the organelle.**

In the first year of Statement of work, we addressed **Specific Aim 1**. We found that a variety of apoptotic inducers, including certain chemicals and chemotherapeutic drugs, seem to kill PCa cells by activating MAP. Several prominent features are associated with this apoptotic pathway. First, apoptosis depends on the mitochondrial respiratory chain (MRC) function and MAP inducers activate MRC early on. Second, soon after apoptotic treatment, there is increased cytochrome c translocation from cytosol to mitochondria.

## KEY RESEARCH ACCOMPLISHMENTS

- 1) We found that apoptosis in multiple cell types induced by a variety of stimuli is preceded by an early induction of MRC proteins such as cytochrome c (which is encoded by a nuclear gene) and cytochrome c oxidase subunit II or COX II (which is encoded by the mitochondrial genome). Several non-MRC proteins that are normally localized in the mitochondria, *e.g.*, Smac, Bim, Bak, and Bcl-2, are also rapidly upregulated.
- 2) The upregulation of many of these proteins (*e.g.*, cytochrome c and COX II) result from transcriptional activation of the respective genes.
- 3) The upregulated cytosolic cytochrome c rapidly translocates to the mitochondria, resulting in a dramatic accumulation of holocytochrome c in the mitochondria, accompanied by increasing holocytochrome c release into the cytosol.
- 4) The increased cytochrome c transport from cytosol to the mitochondria does not depend on the mitochondrial protein synthesis or MRC *per se*. In contrast, cytochrome c release from the mitochondria involves dynamic changes in Bcl-2 family proteins (*e.g.*, upregulation of Bak and cytosolic translocation of Bcl-x<sub>L</sub>), loss of mitochondrial membrane potential ( $\Delta\psi_m$ ), free radical generation, and opening of permeability transition pore (PTP).
- 5) Early induction of MRC proteins occurs prior to caspase activation, PARP cleavage and apoptosis induced by various stimuli in multiple cell types.
- 6) Over-expression of cytochrome c enhances caspase activation and promotes cell death, but simple upregulation of cytochrome c using an ecdysone-inducible system is, by itself, insufficient to induce apoptosis.
- 7) As simple upregulation of cytochrome c using ecdysone inducible system and pCMS-EGFP did not result in an increase in apoptosis, this provided evidence that only upregulation of cytochrome c is not sufficient to induce apoptosis. Hence, we did not make an attempt to generate ecdysone inducible cells expressing antisense cytochrome c.

## FUTURE PLANS

For Specific Aim 2, I have already started my work to find the mechanism involved in activation of caspase-3. My strategies for this year are to:

- 1) Study activation of various caspases upstream and downstream of mitochondrial caspase-3 during MAP induction in PCa using Western blot analysis, caspase activity (substrate cleavage) assays.
- 2) Use various peptide inhibitors to understand the order of activation of different caspases.
- 3) Identify new/novel molecules involved in activation of mitochondrial caspase-3 using proteome analysis.

## REPORTABLE OUTCOMES

### Publication

**Chandra, D.**, Liu, J-W., and Tang, D. G. (2002) Early mitochondria activation and cytochrome c up-regulation during apoptosis. *J. Biol. Chem.* **277**, 50842-50854.

### Paper presented in conference

**Chandra, D.** and Tang, D. G. Mitochondria activation-dependent apoptotic pathway (MADAP): Transcriptional activation of cytochrome c and increased translocation of cytochrome c to mitochondria preceding apoptosis. 93<sup>rd</sup> Annual Meeting of American Association for Cancer Research, San Francisco, California, USA, April 6-10, 2002.

## CONCLUSIONS

Our results suggest that apoptosis induced by many stimuli involves an early mitochondrial activation, which is characterized by up-regulation of the mitochondrial respiratory chain (MRC) proteins (such as cytochrome c and cytochrome c oxidase subunits II) and many other mitochondrially-localized non-MRC proteins (such as Smac, VDAC). A cardinal feature of this mitochondrial activation-dependent apoptotic pathway (or MADAP) is the early up-regulation and enrichment of cyt. c in the mitochondria preceding its release. Mitochondrial activation may be responsible for the subsequent disruption of MRC functions, loss of  $\Delta\psi_m$ , cytochrome c release, and, ultimately, cell death. I finished the proposed work mentioned in the Statement of Work for the first year of my grant, and I am looking forward to completing Specific Aim 2 in the speculated time frame.

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## APPENDIX MATERIAL

### Mitochondrial Respiratory Chain (MRC)-Dependent Apoptotic Pathway: Upregulation of MRC Proteins, Accumulation of Cytochrome c in the Mitochondria, and Lack of Release of Mitochondrial Cytochrome c Prior to Caspase Activation and Apoptosis

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Apoptosis induced by many stimuli requires the mitochondrial respiratory chain (MRC), but the molecular mechanisms underlying this MRC-dependent apoptotic pathway, or MAP, remain unclear. We report here that, during MAP, MRC proteins such as cytochrome c, which is encoded by a nuclear gene, and cytochrome c oxidase subunit II (COX II), which is encoded by the mitochondrial genome, are rapidly upregulated. The upregulation of both cytochrome c and COX II results from transcriptional activation of the respective genes. The upregulated cytosolic cytochrome c rapidly translocates to mitochondria, resulting in a dramatic accumulation of cytochrome c in the mitochondria prior to caspase activation and apoptosis. The increased translocation of cytochrome c from cytosol to the mitochondria does not depend on the mitochondrial protein synthesis or MRC *per se*. Surprisingly, no apparent cytochrome c release is observed when caspases are activated and when cells show apoptotic nuclear morphologies, as revealed by several biochemical and biological methods. Over-expression of cytochrome c enhances caspase activation and promotes cell death triggered by MAP inducers, although simple upregulation of cytochrome c using an ecdysone-inducible system is, by itself, insufficient to induce apoptosis. Taken together, these results suggest that early induction of MRC genes, rapid translocation of cytochrome c to and its accumulation in the mitochondria, and absence of cytochrome c release from mitochondria prior to caspase activation are cardinal features of MAP.

## Early Mitochondrial Activation and Cytochrome *c* Up-regulation during Apoptosis\*<sup>§</sup>

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Apoptosis induced by many stimuli requires the mitochondrial respiratory chain (MRC) function. While studying the molecular mechanisms underlying this MRC-dependent apoptotic pathway, we find that apoptosis in multiple cell types induced by a variety of stimuli is preceded by an early induction of MRC proteins such as cytochrome *c* (which is encoded by a nuclear gene) and cytochrome *c* oxidase subunit II (COX II) (which is encoded by the mitochondrial genome). Several non-MRC proteins localized in the mitochondria, e.g. Smac, Bim, Bak, and Bcl-2, are also rapidly up-regulated. The up-regulation of many of these proteins (e.g. cytochrome *c*, COX II, and Bim) results from transcriptional activation of the respective genes. The up-regulated cytosolic cytochrome *c* rapidly translocates to the mitochondria, resulting in an accumulation of holocytochrome *c* in the mitochondria accompanied by increasing holocytochrome *c* release into the cytosol. The increased cytochrome *c* transport from cytosol to the mitochondria does not depend on the mitochondrial protein synthesis or MRC *per se*. In contrast, cytochrome *c* release from the mitochondria involves dynamic changes in Bcl-2 family proteins (e.g. up-regulation of Bak, Bcl-2, and Bcl-x<sub>L</sub>), opening of permeability transition pore, and loss of mitochondrial membrane potential. Overexpression of cytochrome *c* enhances caspase activation and promotes cell death in response to apoptotic stimulation, but simple up-regulation of cytochrome *c* using an ecdysone-inducible system is, by itself, insufficient to induce apoptosis. Taken together, these results suggest that apoptosis induced by many stimuli involves an early mitochondrial activation, which may be responsible for the subsequent disruption of MRC functions, loss of  $\Delta\psi_m$ , cytochrome *c* release, and ultimately cell death.

Mitochondria generate ATP through the mitochondrial respiratory chain (MRC),<sup>1</sup> which is composed of four multisubunit

respiration complexes (I–IV) and two mobile electron carriers (i.e. cytochrome *c* and ubiquinone). Electrons from reducing substrates such as NADH and succinate are transferred from complex I (NADH dehydrogenase) or complex II (succinate dehydrogenase), respectively, to ubiquinone, to complex III (cytochrome *c* reductase), to cytochrome *c*, to complex IV (cytochrome *c* oxidase or COX), and finally to O<sub>2</sub>. The electron transport through complexes I, III, and IV is accompanied by the pumping of protons from the matrix to the intermembrane space, where the protons establish a mitochondrial membrane potential ( $\Delta\psi_m$ ) by forming a proton and a pH gradient. The reverse flow of the protons from the intermembrane space into the matrix drives another multiprotein complex, F<sub>0</sub>F<sub>1</sub>-ATPase (or complex V), to produce ATP. The protein subunits in complexes I, III, IV, and V are encoded by both nuclear and mitochondrial genomes, thus necessitating smooth communications between and coordinated gene expressions from two genomes (1). Abnormal MRC functions due to genetic defects or chemical disruption, for example, result in a deficiency in ATP generation, leading to cell necrosis (2–4).

Mitochondria also play a pivotal role in regulating another mode of cell death, i.e. apoptosis. Mitochondria generally play a proapoptotic role in most model systems, evoking different mechanisms including ROS production (5–7), Ca<sup>2+</sup> release (8, 9),  $\Delta\psi_m$  collapse (10), opening of the permeability transition pores (PTP) (11, 12), matrix swelling and outer membrane rupture (13), and release of apoptogenic factors including cytochrome *c* (14, 15), apoptosis-inducing factor (16), second mitochondria-derived activator of caspase (Smac/DIABLO; see Refs. 17 and 18), Smac-related serine protease HtrA2 (19–22), endonuclease G (23), and caspases (24, 25). Exactly how these diverse mechanisms converge to activate caspases is still unclear. Two apoptotic pathways are relatively well understood at the molecular level. In the intrinsic (or mitochondrial) pathway, apoptotic signaling somehow impacts mitochondria such that the mitochondrial cytochrome *c* is released into the cytosol, where it binds to the adaptor protein Apaf-1 and procaspase-9, leading to the formation of apoptosome and subse-

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains Figs. 1S–3S.

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<sup>1</sup> The abbreviations used are: MRC, mitochondrial respiratory chain;

A/D, actinomycin D; AFC, 7-amino-4-trifluoromethylcoumarin; BMD188, a hydroxamic acid compound; CHX, cycloheximide; COX II, cytochrome *c* oxidase subunit II; CsA, cyclosporin A; DAPI, 4',6'-diamidino-2-phenylindole; GFP, green fluorescence protein; MADAP, mitochondrial activation-dependent apoptotic pathway; PARP, poly-(ADP-ribose) polymerase; PTP, permeability transition pore; ROS, reactive oxygen species; RT, reverse transcriptase; VDAC, voltage-dependent anion channel; VP16, etoposide;  $\rho^0$  cells, respiration-deficient cells; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; AFC, 7-amino-4-trifluoromethylcoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; z, benzyloxycarbonyl; fmk, fluoromethyl ketone; FBS, fetal bovine serum; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; MAC, mitochondria apoptosis-induced channel; NAC, N-acetylcysteine.



quent activation of "executioner" caspases such as caspase-3, -6, or -7 (26). In the extrinsic pathway, engagement of the cell surface death receptors (e.g., Fas, TNFR1, DR-3, DR-4, and DR-5) results in the activation of initiator caspase-8 through adaptor proteins such as FADD and TRADD (27), which then activates downstream executioner caspases. The death receptor pathway can be amplified by the mitochondrial pathway either through the translocation of tBid, a caspase 8 cleavage product of Bid, to the mitochondria, which induces cytochrome c release (28, 29), or through the mitochondrial release of Smac, which neutralizes the inhibitory effect of inhibitor of apoptosis proteins on caspases (17, 18, 30).

Apoptosis induced by many stimuli seems to depend on MRC. For example, MRC function has been reported to be required for apoptosis induced by TNF- $\alpha$  (31–33), lipoxygenase inhibitor nordihydroguaiaretic acid (34), butyrate, and some other short chain fatty acids (35), ceramide (36), hydroxamic acid compound BMD188 (37), manganese (38), synthetic retinoid CD437 (39), O<sub>2</sub> deprivation (40), oxidants such as *tert*-butylhydroperoxide (41) and hydrogen peroxide (42), and Ca<sup>2+</sup> overloading (43). Thus, in these apoptotic model systems, MRC-deficient  $\rho^0$  cells are more resistant to apoptosis, and MRC inhibitors (such as rotenone and antimycin A) block or inhibit apoptosis. Also in support of the dependence of apoptosis on MRC, complex I deficiency in leukemia cells results in apoptosis resistance (44). Deficiency in complex IV in colon carcinoma cells renders them resistant to apoptosis induction (35). Similarly, F<sub>0</sub>F<sub>1</sub>-ATPase is required for Bax-induced apoptosis (45).

The molecular mechanisms underlying this MRC-dependent apoptosis remain unclear. Here we report that apoptosis induced by BMD188, a chemical that causes cell death in an MRC-dependent manner (37), involves an early up-regulation of MRC proteins (in particular, cytochrome c) prior to caspase activation and cell death. Importantly, this phenomenon seems to represent a general early apoptotic response as it is also observed in multiple cell types induced to die by a variety of stimuli.

#### MATERIALS AND METHODS

##### Cells and Reagents

Human osteosarcoma cells 143B(TK<sup>-</sup>) (143B) and fibroblasts GM701.2-8C (GM701) and their respective mtDNA-less, respiration-deficient  $\rho^0$  derivatives 143B206 and GM701.2-8C (2) cells (46) were kindly provided by Dr. M. King (Thomas Jefferson University). 143B and GM701 cells were cultured in Dulbecco's minimum essential medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. 143B206 and GM701.2-8C (2) cells were cultured in Dulbecco's minimum essential medium with high glucose supplemented with 100  $\mu$ M pyruvate, 200 ng/ml ethidium bromide, 50  $\mu$ M uridine, 10% FBS, and antibiotics. Human prostate cancer cells, PC3 and LNCaP, and breast carcinoma cells, MDA-MB231, were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 supplemented with 5 and 10% FBS, respectively. The  $\rho^0$  PC3 clone 6 cells (37) were cultured as for 143B206 cells.

The primary antibodies used were listed in Table I. Secondary antibodies, i.e., goat, donkey, or sheep anti-mouse or rabbit IgG conjugated to horseradish peroxidase, fluorescein isothiocyanate, or rhodamine, together with ECL (enhanced chemiluminescence) reagents were acquired from Amersham Biosciences. Fluorogenic caspase substrates DEVD-AFC and LEHD-AFC, pan-caspase inhibitor z-VAD-fmk, and caspase-3/6/7 inhibitor, z-DEVD-fmk, were bought from Biomol (Plymouth Meeting, PA). Ponasterone and Zeocin were purchased from Invitrogen. Annexin V conjugated to AlexaFluor 568 and mitochondrial dyes were purchased from Molecular Probes (Eugene, OR). Liposome FuGENE 6 was bought from Roche Molecular Biochemicals. All other chemicals were purchased from Sigma unless specified otherwise.

##### Subcellular Fractionation and Western Blotting

Mitochondria were prepared using differential centrifugation (37, 47, 48) with slight modifications. Briefly, cells were treated with various chemicals or vehicle (ethanol or Me<sub>2</sub>SO) control. In some experiments,

cells were pretreated with protein synthesis inhibitor cycloheximide (CHX), RNA synthesis inhibitor actinomycin D (A/D), or mitochondrial protein synthesis inhibitor tetracycline before apoptosis induction. At the end of the treatment, cells were harvested by scraping, washed twice in ice-cold PBS, and resuspended in 600  $\mu$ l of homogenizing buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium EDTA, 1 mM sodium EGTA, and 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 mM leupeptin, 1  $\mu$ g/ml pepstatin A, and 1  $\mu$ g/ml chymostatin). After 30 min of incubation on ice, cells were homogenized in the same buffer using a glass Pyrex homogenizer (type A pestle, 140 strokes). Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at 500  $\times$  g for 5 min at 4 °C. The resulting supernatant was centrifuged at 10,000  $\times$  g for 20 min to obtain mitochondria. The remaining supernatant was again subjected to centrifugation at 100,000  $\times$  g for 1 h to obtain the cytosolic fraction (i.e., S100 fraction). The mitochondrial pellet was washed three times in homogenizing buffer, and then solubilized in TNC buffer (10 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40, 5 mM CaCl<sub>2</sub>) containing protease inhibitors. Protein concentration was determined by Micro-BCA kit (Pierce).

For Western blotting, 25  $\mu$ g of proteins (mitochondrial or cytosolic fractions) was loaded in each lane of a 15% SDS-polyacrylamide gel. After gel electrophoresis and protein transfer, the membrane was probed or reprobed, after stripping, with various primary and corresponding secondary antibodies (37, 47). Western blotting was performed using ECL as described previously (37, 47).

##### Measurement of Apoptosis

Apoptosis was measured using several biochemical and biological approaches.

**PARP Cleavage**—PARP cleavage assays were performed as described previously (37, 47).

**Caspase-3 Activation**—Caspase-3 cleavage (activation) was analyzed by Western blotting. Cells were lysed in TNC lysis buffer, and 100  $\mu$ g of whole cell lysates was separated on 15% SDS-PAGE. After protein transfer, the blot was probed with a monoclonal antibody for caspase-3. The activation of caspase-3 was monitored by a decrease or disappearance of the ~32-kDa procaspase-3 (37).

**DEVDase and LEHDase Activity**—Cells were washed twice in PBS, and the whole cell lysates were made in the lysis buffer (50 mM HEPES, 1% Triton X-100, 0.1% CHAPS, 1 mM dithiothreitol, and 0.1 mM EDTA). Forty  $\mu$ g of protein was added to a reaction mixture containing 30  $\mu$ M fluorogenic peptide substrates, DEVD-AFC or LEHD-AFC, 50 mM of HEPES, pH 7.4, 10% glycerol, 0.1% CHAPS, 100 mM NaCl, 1 mM EDTA, and 10 mM dithiothreitol, in a total volume of 1 ml and incubated at 37 °C for 1 h. Production of 7-amino-4-trifluoromethylcoumarin (AFC) was monitored in a spectrofluorimeter (Hitachi F-2000 fluorescence spectrophotometer) using excitation wavelength 400 nm and emission wavelength 505 nm. The fluorescent units were converted into nanomoles of AFC released per h per mg of protein using a standard curve. The results were generally presented as fold activation over the control.

**DNA Fragmentation**—Fragmented DNA was extracted using SDS/RNase/proteinase K methods (37, 47), and 20  $\mu$ g of DNA was run on 1.2% agarose gel.

**Quantification of Apoptotic Nuclei Using DAPI Staining**—Cells were plated on glass coverslips (4  $\times$  10<sup>4</sup> cells/18-mm<sup>2</sup> coverslip) and the next day treated with vehicle control (i.e., ethanol or Me<sub>2</sub>SO) or various inducers. Thereafter, cells were incubated live with DAPI (0.5  $\mu$ g/ml) for 10 min at 37 °C followed by washing. The percentage of cells exhibiting apoptotic nuclei, as judged by chromatin condensation or nuclear fragmentation, was assessed by fluorescence microscopy (49). An average of 600–700 cells was counted for each condition.

##### Immunofluorescence Analysis of Cytochrome c Distribution, Mitochondrial Membrane Potential, and Apoptosis

Cells grown on glass coverslips were treated for various time intervals. Fifteen min prior to the end of the treatment, cells were incubated live with MitoTracker Orange CMTMRos to label mitochondria (37). Then cells were fixed in 4% paraformaldehyde for 10 min followed by permeabilization in 1% Triton X-100 for 10 min. After washing in PBS, cells were first blocked in 20% goat whole serum for 30 min at 37 °C and then incubated with monoclonal anti-cytochrome c antibody (clone 6H2.B4, 1:500) for 1 h at 37 °C. Finally, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:1000) for 1 h at 37 °C. After thorough washing, coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories, Inc.,

Burlingame, CA) and observed under an Olympus BX40 epifluorescence microscope. Images were captured with MagnaFire software and processed in Adobe Photoshop. In a separate set of samples, following apoptotic treatments, cells were washed once with 1× PBS and then incubated in the Annexin-Binding Buffer containing annexin V-Alexa-Fluor conjugates for 30 min followed by washing. Images were captured on an Olympus IX50 inverted fluorescence microscope.

#### RT-PCR Analysis of Cytochrome *c* and COX II mRNA Expression

Total RNA was isolated using Tri-Reagent (Invitrogen). RT was performed using 2 µg of total RNA (at 42 °C for 2 h) in a total 20-µl reaction volume containing random hexamers and Superscript II reverse transcriptase (Invitrogen). PCR primers, designed based on the published cytochrome *c* and COX II cDNA sequences, are 5'-TTTGAATCCCAATGGGTGATGTGAG-3' (cytochrome *c*, sense), 5'-TTTGAATTCCTCATAGTAGCTTTTGTAG-3' (cytochrome *c*, antisense), 5'-CCATCCCTACGCATCCTTTAC-3' (COX II, sense), and 5'-GTTTGCTCCACAGATTTCAGAG-3' (COX II, antisense). For PCR, 1 µl of cDNA was used in a 25-µl reaction containing 0.5 µM primers, dNTPs and Taq, using the cycling profile 94 °C × 45 s, 56 °C × 1 min, and 72 °C × 1 min for 14 cycles for COX II and 23 cycles for cytochrome *c* with a final extension at 72 °C for 10 min. PCR products were analyzed on 1% agarose gel. RT-PCR of glyceraldehyde 3-phosphate dehydrogenase (50) was used as a control.

#### Transient Transfection with pEGFP-Cytochrome *c*

The pEGFP-cytochrome *c* expression plasmid, in which the full-length rat cytochrome *c* cDNA was cloned into the *NheI* and *XhoI* sites of the pEGFP-N1 (Clontech), was kindly provided by Dr. A.-L. Nieminen (Case Western Reserve University; see Ref. 51). GM701 cells, plated 1 day earlier on 10-cm culture dishes to achieve 50–60% confluence, were transfected, using FuGENE 6, with 15 µg of either empty vector alone or pEGFP-cytochrome *c*. Twenty four h after transfection, cells were treated with BMD188 for various times and then harvested and used for subcellular fractionations as described above. To assess apoptosis, GM701 cells grown on coverslips were transfected with 1 µg of plasmids. Twenty four h later, cells were treated with BMD188 followed by DAPI staining as described above. The percentage of GFP-positive and -negative cells with apoptotic nuclear morphology was determined by fluorescence microscopy (49). At least 600–700 cells were counted for each condition.

#### Up-regulation of Cytochrome *c* Using the Ecdysone-inducible System

Plasmids pVgRXX and pIND were obtained from Invitrogen. pIND/cyt-*c*-GFP was prepared by cloning the *NheI*/*NotI* fragment of the rat cytochrome *c*-GFP fusion cDNA from pEGFP-cytochrome *c* (51) into pIND/V5-His-B, whereas pIND-GFP was prepared by cloning the EGFP fragment (from pEGFP-N1) between the *HindIII* and *NotI* sites of pIND/Hygro (courtesy of Dr. T.-J. Liu, MD Anderson Cancer Center, Houston, TX). Ecdysone-inducible cell lines were generated in two steps. First, GM701 cells were transfected with pVgRXX by electroporation (1025 microfarads, 260 V in a Bio-Rad apparatus). Stable transfectants were selected with Zeocin (500 µg/ml) for 3–4 weeks and stable clones isolated using a cloning ring. The stable clones, named EcR-GM701, were characterized by GFP expression in response to ponasterone (2 µM) after transient transfection with pIND-GFP. EcR-293 cells were kindly provided by Dr. M. Bedford. In the second step, EcR-GM701 and EcR-293 cells were electroporated with pIND/cyt-*c*-GFP or pIND/GFP followed by G418 (1 mg/ml) selection. Stable clones were picked by ring cloning and screened by immunoblotting using antibody against GFP upon ponasterone (2 µM) induction. GM701-pIND/cyt-*c*-GFP, GM701-pIND/GFP, 293-pIND/cyt-*c*-GFP, and 293-pIND/GFP cells were maintained in Zeocin (500 µg/ml) and G418 (1 mg/ml)-containing medium. These cells were used in subcellular fractionation and apoptosis studies upon treatment with ponasterone for various times.

#### Up-regulation of Cytochrome *c* Using the pCMS-EGFP Expression System

Full-length cDNA sequence of human cytochrome *c* was synthesized by PCR amplification using Taq polymerase (Eppendorf, Germany) and cloned between the *EcoRI* and *XbaI* sites of pCMS-EGFP vector (Clontech). The Kozak sequence was introduced in sense primer. The resultant plasmid was sequenced and designated as pCMS-EGFP/cyt-*c*, in which cytochrome *c* and EGFP are synthesized from two separate

promoters. GM701 fibroblasts were either untransfected or transfected with the empty vector (pCMS-EGFP) or pCMS-EGFP/cyt-*c*. Twenty-four h after transfection, cells were harvested, and mitochondrial and cytosolic fractions were prepared, and 25 µg/lane of protein from each sample was separated by 15% SDS-PAGE. Following protein transfer, the membranes were probed and reprobed with antibodies against cytochrome *c*, GFP, actin, or HSP60, as indicated under "Results." Apoptosis was quantified using the DAPI staining 24 h after transfection.

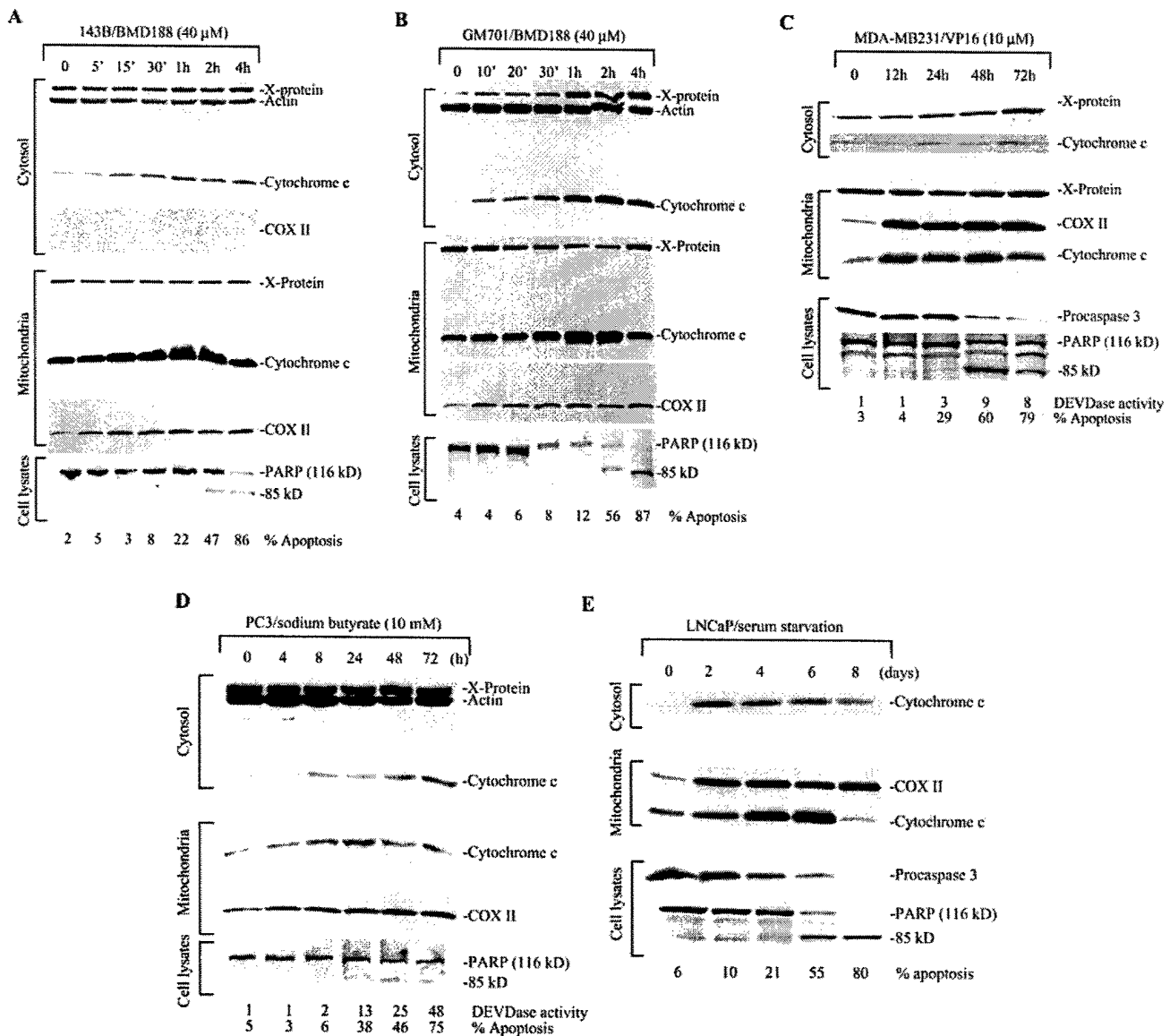
#### Statistical Analysis

The statistical significance between experimental groups was determined by Student's *t* test using the SPSS-10 software. Differences at *p* < 0.05 were considered statistically significant.

#### RESULTS

**Multiple Apoptotic Stimuli Induce an Increase of MRC Proteins Such as COX II and Cytochrome *c* Early during Apoptosis Induction**—We demonstrated previously (37) that apoptosis of epithelial cancer cells induced by chemical BMD188 requires the MRC function. Thus, *p*<sup>0</sup> PC3 (prostate cancer) cells or PC3 cells whose MRC had been blocked by various respiration complex-specific inhibitors were more resistant to BMD188-induced apoptosis (37). Further experiments demonstrate that BMD188 induces apoptosis in many other cell types, *e.g.* 143B osteosarcoma cells (Supplemental Material Fig. 1S) and GM701 fibroblasts (not shown) also in an MRC-dependent manner. To explore the molecular mechanisms of this MRC-dependent apoptotic pathway, we purified mitochondrial and S100 cytosolic fractions and used them in quantitative Western blotting to examine the protein levels of two essential MRC components, cytochrome *c*, which is encoded by the nuclear gene, and COX II, which is encoded by the mitochondrial genome. The protein levels were then correlated with caspase activation, which is measured by DEVDase activity, caspase-3 activation, or PARP cleavage, and with apoptosis, which is quantitated by counting apoptotic nuclei (see details under "Materials and Methods").

By using the above approach, we found that treatment of 143B cells with BMD188 induced a rapid (within 5 min) increase in the protein level of COX II (Fig. 1A). COX II protein was never detected in the cytosolic fractions in all of our experiments (Fig. 1A and not shown), suggesting that there was no substantial mitochondrial damage during subcellular fractionations and that the cytosolic fractions were not contaminated with the mitochondrial proteins. The reciprocal experiments measuring the lactate dehydrogenase (a cytosolic protein) activity also indicated that there was minimal contamination of the mitochondrial fractions with the cytosolic proteins (not shown). The COX II protein level plateaued at 30 min to 1 h after BMD188 treatment and slightly declined thereafter (Fig. 1A). Interestingly, 15 min post-BMD188 treatment, the total cytochrome *c* protein level also increased, in both cytosol and mitochondria (Fig. 1A), as revealed by monoclonal antibody against cytochrome *c* clone 7H8.2C12, which recognizes both apocytochrome *c* (*i.e.* cytochrome *c* in the cytosol without heme attached) and holocytochrome *c* (*i.e.* cytochrome *c* in the mitochondria with heme attached). (Note that, depending on the respiration status, a small amount of cytochrome *c* is often detected in the cytosol of untreated cells.) The cytochrome *c* levels continued to increase until ~1 h when an ~4-fold increase of cytochrome *c* was detected in both cytosol and mitochondria, and 22% of the cells were apoptotic (Fig. 1A). When ~50% 143B cells were apoptotic and prominent PARP cleavage (an indication of caspase activation) was observed at 2 h, cytochrome *c* levels in both compartments slightly decreased (Fig. 1A). Note that PARP cleavage becomes detectable only after sufficient numbers of cells have undergone apoptosis (*e.g.* see Fig. 1, A–E). By 4 h when most PARP was cleaved and ~90% of



**FIG. 1. Up-regulation of MRC proteins early during apoptosis induction.** Western blot analysis of cytosolic and mitochondrial fractions from 143B cells treated with BMD188 (A), GM701 cells treated with BMD188 (B), MDA-MB-231 cells treated with VP16 (C), PC3 cells treated with sodium butyrate (D), and LNCaP cells treated with serum starvation (E). Mitochondrial and cytosolic fractions were prepared, and 25  $\mu$ g of proteins (cytosolic or mitochondrial fractions) from each sample was used in Western blotting for cytochrome c, COX II, and actin. Several different biochemical (PARP cleavage, caspase-3 activation, and DEVDase activity) and biological (*i.e.* apoptotic nuclear morphology upon DAPI labeling of live cells) methods were used, in different combinations, to assess apoptosis (see "Materials and Methods"). In some samples (*e.g.* A, B, and D), the cytosol blots were incubated with a mixture of antibodies to both cytochrome c and actin. Data shown are representative of 3–5 independent experiments.

the cells were apoptotic, significant amounts of mitochondrial cytochrome c similar to the basal level and of cytosolic cytochrome c higher than the basal level were still observed (Fig. 1A). Note that 7H8.2C12 also detected an ~50-kDa protein labeled as X-protein, whose identity remains unclear (37, 47, 52).

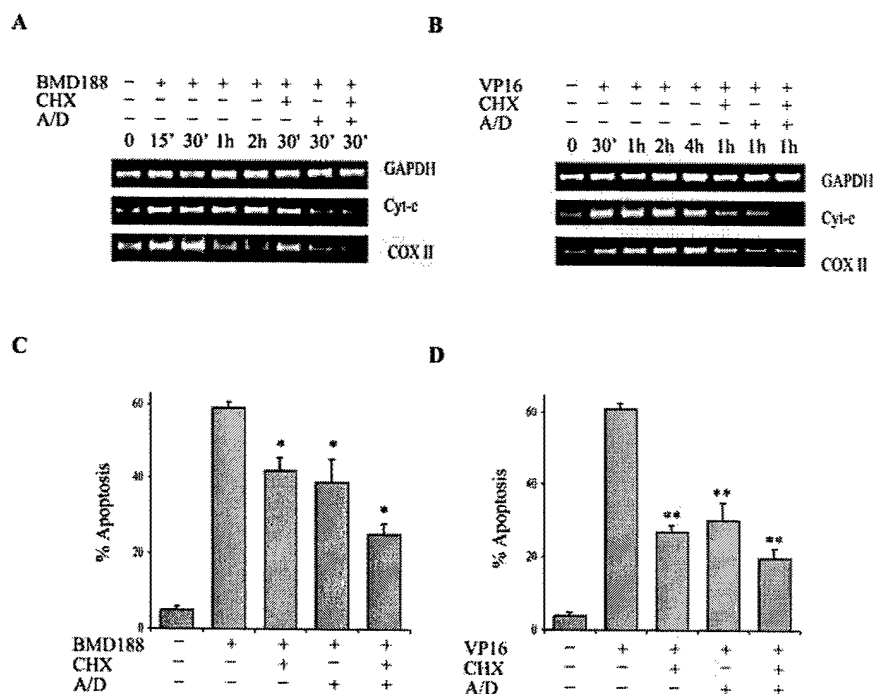
Similarly, treatment of GM701 fibroblasts with BMD188 rapidly up-regulated COX II as well as cytochrome c (Fig. 1B). Increased COX II was detected at 10 min and plateaued by 1 h after drug treatment (Fig. 1B). Increased cytochrome c was observed in both cytosol (~3-fold) and mitochondria (~1.5-fold) in the first 10–20 min, and by 30 min the increase became more obvious (5-fold in cytosol and 2.5-fold in mitochondria) (Fig. 1B). By 1 h, when ~12% cells were apoptotic, peak levels of cytochrome c in both cytosol (10-fold increase) and mitochondria (4-fold increase) were observed (Fig. 1B). By 2 h when ~60% of the cells was apoptotic and PARP was cleaved, mito-

chondrial cytochrome c began to decrease, whereas the cytosolic cytochrome c level remained about the same (Fig. 1B). By 4 h when ~90% of cells were apoptotic and PARP was nearly completely cleaved, cytochrome c levels in both cytosol and mitochondria decreased (Fig. 1B). As observed previously (37) in PC3 cells, in both 143B and GM701 cells treated with BMD188, apoptosis could be dose-dependently inhibited by z-VAD-fmk, a pan-caspase inhibitor, as well as by z-DEVD-fmk, a caspase-3/6/7 inhibitor (not shown), suggesting that the cell death is caspase-dependent. Collectively, data presented in Fig. 1, A and B, together with our previous observations showing early induction of COX I (encoded by mitochondrial genome) and COX IV (encoded by the nuclear genome) (37), suggest that there is an early induction of MRC proteins during apoptosis induced by BMD188, an agent whose pro-apoptotic effect depends on MRC (37).

To determine whether this early induction of MRC proteins

**FIG. 2. Transcriptional activation of cytochrome *c* and COX II genes and inhibition of apoptosis by transcription and translation inhibitors.**

**A and B,** transcriptional up-regulation of cytochrome *c* (Cyt-*c*) and COX II. **A,** GM701 fibroblast cells were treated with BMD188 (40  $\mu$ M); or **B,** MDA-MB-231 cells were treated with VP16 (10  $\mu$ M) for the time intervals indicated. Some samples were pretreated with cycloheximide (CHX, 1  $\mu$ M), actinomycin D (A/D, 1 nM), or both for 1 h before apoptotic stimulation. RT-PCR was performed as detailed under "Materials and Methods." Data are representative of at least five separate experiments. **C and D,** inhibition of apoptosis by protein and RNA synthesis inhibitors. GM701 (**C**) or MDA-MB-231 (**D**) cells grown on glass coverslips were treated with BMD188 (40  $\mu$ M, 2 h) or VP16 (10  $\mu$ M, 48 h), respectively, either alone or in the presence of CHX, A/D, or both as indicated. The percentages of apoptotic cells upon DAPI staining were enumerated under a fluorescent microscope. Values represent the mean  $\pm$  S.D. from five separate experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (Student *t* test) compared with cells treated BMD188 (**C**) or VP16 (**D**) alone.



is restricted only to MRC-dependent apoptotic stimuli, we carried out similar experiments in multiple cell types with a variety of apoptotic inducers of different mechanisms of action. First, we treated MDA-MB231 breast cancer cells with VP16 (etoposide), a commonly used chemotherapeutic drug that inhibits DNA topoisomerase II. As shown in Fig. 1C, maximum up-regulations of COX II and cytochrome *c* occurred as early as 12 h after drug treatment. Nearly all up-regulated cytochrome *c* was present in the mitochondria in MDA-MB231 cells treated with VP16 (Fig. 1C), which is different from 143B or GM701 cells treated with BMD188 (Fig. 1, A and B). By 12 h no caspase activation and significant death were detected (Fig. 1C). By 24 h caspases (*i.e.* DEVDase) were activated ~3-fold, and ~30% of the cells were apoptotic (Fig. 1C). By 48 h after the drug treatment when obvious caspase-3 cleavage and DEVDase activity were detected, PARP was cleaved, and 60% of the cells were apoptotic, maximum levels of cytochrome *c* and COX II were still observed in the mitochondria (Fig. 1C). By 72 h when nearly 80% of the cells were apoptotic and prominent caspase-3 cleavage occurred, cytochrome *c* in the mitochondria decreased but the COX II protein level remained about the same (Fig. 1C). There was a slight increase of cytochrome *c* in the cytosol 24–72 h after VP16 treatment (Fig. 1C). Apoptosis in MDA-MB-231 cells was dose-dependently inhibited by z-VAD-fmk or z-DEVD-fmk (not shown), suggesting that the VP16-induced cell death is also caspase-dependent.

Likewise, treatment of PC3 cells with butyrate, a short chain fatty acid inhibitor of histone deacetylase (35), resulted in a time-dependent up-regulation of COX II and cytochrome *c* in both cytosolic and mitochondrial compartments (Fig. 1D). By 24 h post-treatment when caspase activity increased by 13-fold, PARP was cleaved, and ~40% of the cells were apoptotic, the maximum amount of cytochrome *c* accumulated in the mitochondria (Fig. 1D). Starting from 48 h on, the mitochondrial cytochrome *c* began to decrease accompanied by an increase in the cytosolic cytochrome *c* (Fig. 1D). Similarly, in LNCaP cells subjected to serum starvation, maximum induction of COX II was observed by day 2, after which the COX II protein remained at a similar level up to day 10 (Fig. 1E and data not shown). By contrast, a time-dependent up-regulation of cytochrome *c* in the mitochondria was observed (Fig. 1E). Two days

after withdrawal of serum, increased cytochrome *c* in the mitochondria and maximum level of cytochrome *c* in the cytosol were observed (Fig. 1E). There was slightly increased pro-caspase-3 cleavage and cell death at this time point (Fig. 1E). By 4 days when further increased caspase-3 activation and cell death were observed, the mitochondrial cytochrome *c* continued to increase while the cytosolic cytochrome *c* slightly decreased. By 6 days when obvious caspase-3 activation, PARP cleavage, and apoptosis were observed, the mitochondrial cytochrome *c* reached peak level, whereas the cytosolic cytochrome *c* level remained unchanged (Fig. 1E). By 8 days when caspase-3 and PARP were completely cleaved and 80% of the cells apoptotic, the mitochondrial cytochrome *c* was almost completely lost, and the cytosolic cytochrome *c* also decreased (Fig. 1E).

Similar studies in PC3 cells treated with camptothecin and DLD-1 (colon cancer) cells treated with herbimycin also revealed rapid up-regulations of cytochrome *c* and COX II (not shown). Altogether, these data demonstrate that early up-regulation of MRC proteins such as COX II and cytochrome *c* represents a common early event in apoptosis induced by diverse stimuli.

**Increased Cytochrome *c* and COX II Proteins Result from Transcriptional Activation**—Cytochrome *c* is a cytosolic protein encoded by a nuclear gene. Upon synthesis, depending on the metabolic status of the cells, the majority or a fraction of the apocytochrome *c* rapidly translocates to the mitochondria to participate in the electron transport (1). Within mitochondria, apocytochrome *c* is "anchored" to cytochrome *c* heme lyase and turns into holocytochrome *c* upon heme binding (1, 53). Therefore, the increased cytochrome *c* protein levels in both cytosol and mitochondria (Fig. 1, A–E) suggest an increased transcription and/or translation. To test this possibility, we used semi-quantitative RT-PCR to analyze the mRNA levels of cytochrome *c* and COX II during the early phase of apoptosis induction by some of the inducers used in Fig. 1. As shown in Fig. 2A, treatment of GM701 cells with BMD188 resulted in a maximum increase in cytochrome *c* mRNA as early as 15 min, which remained elevated at similar levels until 2 h. Similarly, maximum induction of COX II mRNA was also observed at 15 min post-BMD188 treatment, which decreased by 1 h (Fig. 2A).

TABLE I  
Primary antibodies used in this study

| Ab <sup>a</sup> | Type                       | Source (catalog no.)                     | Remarks  |
|-----------------|----------------------------|--|--|
| Actin           | Mouse mAb                  | ICN (69100)                              |  |
| Bad             | Mouse mAb                  | Santa Cruz Biotechnology (sc-8044)       |  |
| Bak             | Rb pAb                     | Santa Cruz Biotechnology (sc-832)        |  |
| Bak             | Rb pAb                     | Upstate Biotechnology (06-536)           | Recognizes activated Bak                             |
| Bax             | Rb pAb                     | Santa Cruz Biotechnology (554104)        |  |
| Bax             | Rb pAb                     | Upstate Biotechnology (06-499)           | Recognizes activated Bax                             |
| Bcl-2           | Mouse mAb                  | Pharmingen (14831A)                      |  |
| Bcl-x           | Rb pAb                     | Pharmingen (610211)                      |  |
| Bid             | Rb pAb                     | Dr. X Wang                               | Recognizes both t Bid and uncleaved Bid              |
| Bim             | Rb pAb                     | Calbiochem (202000)                      |  |
| COX I           | Mouse mAb                  | Molecular Probes (A-6403)                |  |
| COX II          | Mouse mAb                  | Molecular Probes (A-6404)                |  |
| COX IV          | Mouse mAb                  | Molecular Probes (A-6431)                |  |
| Cyt-c           | Mouse mAb (clone 6H2.B4)   | Pharmingen (556432)                      | Recognizes holocytochrome c on immunofluorescence    |
| Cyt-c           | Mouse mAb (clone 7H8.2C12) | Pharmingen (65981A)                      | Recognizes apo- and holocytochrome c on Western blot |
| Cyt-c           | Mouse mAb                  | R & D Systems (6380-MC-100)              | Recognizes holocytochrome c only on Western blot     |
| Caspase-3       | Mouse mAb                  | Transduction Laboratories (C31720)       | Recognizes the proform only                          |
| Caspase-9       | Rb pAb                     | Chemicon (AB16970)                       |  |
| GFP             | Mouse mAb                  | Clontech (8362-1)                        |  |
| PARP            | Rb pAb                     | Roche Molecular Biochemicals (1 835 238) |  |
| Smac            | Rb pAb                     | Dr. X. Wang                              |  |
| HSP60           | Mouse mAb                  | Chemicon (mAb3514)                       |  |

<sup>a</sup> The abbreviations used are: Ab, antibodies; COX I/II/IV, cytochrome c oxidase subunit I/II/IV; cyt-c, cytochrome c; GFP, green fluorescence protein; PARP, poly-(ADP-ribose); polymerase; mAb, monoclonal antibody; pAb, polyclonal antibody; Rb, rabbit; HSP60, heat shock protein 60.

The induction of both cytochrome *c* and COX II mRNAs was inhibited by A/D, which inhibits RNA transcription. CHX, a protein synthesis inhibitor, did not significantly affect cytochrome *c* mRNA level but significantly inhibited the mRNA of mitochondria-encoded COX II (Fig. 2A). The combination of A/D and CHX resulted in slightly greater inhibition of the cytochrome *c* and COX II mRNAs than either inhibitor alone (Fig. 2A). As expected, CHX and A/D, either individually or in combination, inhibited BMD188 up-regulated COX II and cytochrome *c* proteins in GM701 cells (not shown). Similar RT-PCR analysis was carried out in MDA-MB231 cells treated with VP16. As shown in Fig. 2B, the cytochrome *c* mRNA was maximally induced as early as 30 min post-treatment, which stayed up-regulated at similar levels up to 4 h, the longest time interval examined. In contrast, COX II mRNA was up-regulated in a time-dependent manner, and the peak-level induction was observed at 4 h (Fig. 2B). CHX significantly inhibited both cytochrome *c* and COX II mRNA expression, and A/D demonstrated a more prominent inhibitory effect (Fig. 2B), which is different from BMD188-treated GM701 cells. The combination of CHX and A/D inhibited their mRNA up-regulations more significantly than either treatment alone (Fig. 2B). Together, these results suggest that the up-regulated COX II and cytochrome *c* proteins early during apoptosis induction (Fig. 1, B and C) resulted from an increased mRNA transcription. That CHX also inhibited the mRNA expression of COX II and/or cytochrome *c* (Fig. 2, A and B) is probably due to inhibition of the transcriptional machinery by CHX.

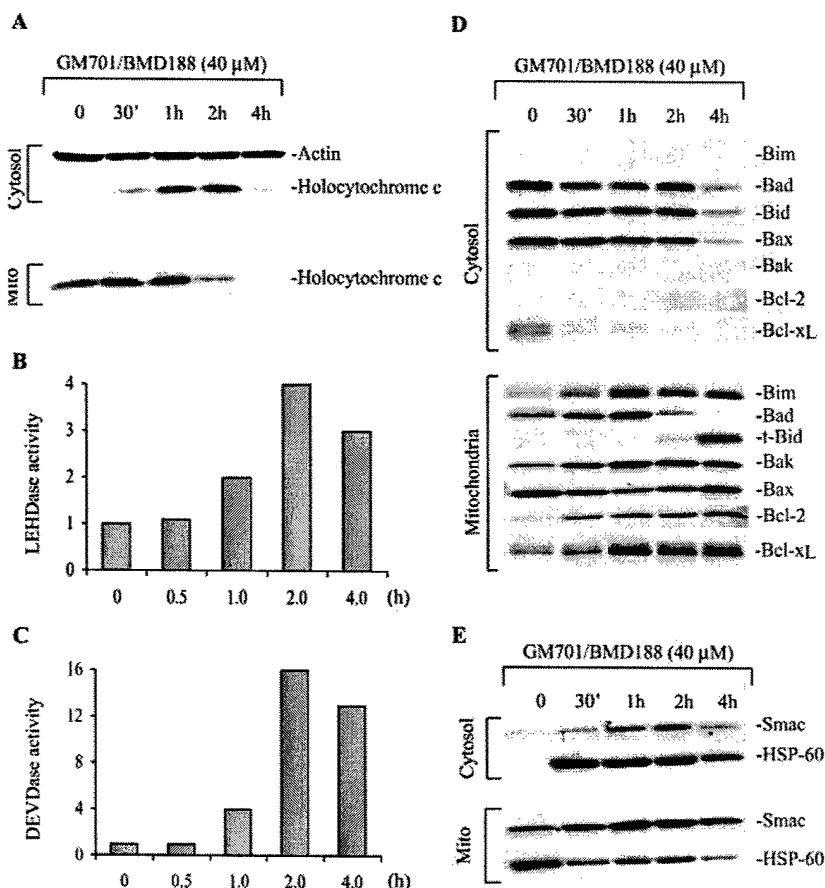
To assess the potential importance of *de novo* RNA and/or protein synthesis in the above apoptotic models, GM701 (Fig. 2C) and MDA-MB-231 (Fig. 2D) cells were pretreated with A/D, CHX, or both 1 h before treating with BMD188 and VP16, respectively. The results indicate that pretreatment of cells with these inhibitors significantly inhibited apoptosis, and the inhibition was more pronounced in VP16-treated MDA-MB231 cells. In both cases, the combination of A/D and CHX resulted in greater inhibition of apoptosis than either inhibitor alone (Fig. 2, C and D).

**Cytochrome *c* Up-regulation in Relation to Cytochrome *c* Release and Caspase Activation**—The preceding experiments demonstrate that multiple apoptotic stimuli, perhaps through transcriptional activation, up-regulate cytochrome *c*, leading to increased cytochrome *c* protein levels in both mitochondria and cytosol. In the intrinsic apoptotic pathway, mitochondrial holocytochrome *c* is released into the cytosol to trigger caspase activation, and only the holo- but not apocytochrome *c* has the apoptogenic effect (14). The anti-cytochrome *c* antibody (*i.e.* 7H8.2C12) utilized in the experiments of Fig. 1 recognizes both apo- and holocytochrome *c* (Table I). Consequently, we could not determine whether the increased cytochrome *c* in the cytosol (Fig. 1, A–E) represents the newly synthesized apocytochrome *c*, mitochondrially released holocytochrome *c*, or a mixture of both. R & D Systems has recently developed an antibody that specifically recognizes holocytochrome *c* (Table I). We thus took advantage of this antibody and utilized BMD188 as the primary apoptotic inducer to address the relationships among cytochrome *c* up-regulation, cytochrome *c* release, and caspase activation.

In untreated GM701 cells holocytochrome *c* existed only in the mitochondria (Fig. 3A). Fifteen min post-BMD188 treatment, slightly increased holocytochrome *c* was observed in the mitochondria, but no holocytochrome *c* could be detected in the cytosol (not shown). By 30 min, there was a substantial increase in the mitochondrial holocytochrome *c* accompanied by a small amount of holocytochrome *c* release into the cytosol. By 1 h, the mitochondrial holocytochrome *c* level remained about the same as at 30 min, but the holocytochrome *c* level in the cytosol dramatically increased (Fig. 3A). By 2 h, the majority of holocytochrome *c* was released from the mitochondria, whereas the holocytochrome *c* level in the cytosol remain about the same as at 1 h (Fig. 3A). By 4 h, the holocytochrome *c* in both mitochondria and cytosol could hardly be detected. These changes in holocytochrome *c* were confirmed by fluorescence microscopy (see Fig. 4). Combined with the data presented in Fig. 1B and 2A, the following scenario can be presented to explain the cytochrome *c* movement. Early (*i.e.* within 10–15



**FIG. 3. Cytochrome *c* alterations in relation to caspase activation (A–C), changes in Bcl-2 family proteins (D), and release of mitochondrial proteins larger than cytochrome *c* (E). A, GM701 cells were treated with BMD188, and mitochondrial (Mito) and cytosolic fractions were prepared as described under “Materials and Methods.” Forty  $\mu$ g of cytosolic or mitochondrial proteins from each sample was used in Western blotting for holocytochrome *c* together with actin. B and C, whole cell lysates (40  $\mu$ g) were used to measure LEHDase activity (B) and DEVDase activity (C), respectively. D and E, the same blot as shown in A was stripped and sequentially reprobed for various Bcl-2 family proteins (D) or for Smac and HSP60 (E). Note that essentially identical results were obtained for Bak and Bax when using antibodies that recognize the conformationally active proteins (Table I; data not shown). Data shown are representative of 3–5 independent experiments.**



min) post-BMD188 treatment, cytochrome *c* is transcriptionally up-regulated resulting in increased cytochrome *c* protein, part of which is transported into the mitochondria and part of which is retained in the cytosol. By 30 min, continued cytochrome *c* up-regulation leads to further increased holocytochrome *c* enrichment in the mitochondria and, at the same time, a low level of mitochondrial holocytochrome *c* begins to be released. By 1 h, the total cytochrome *c* level (as detected by 7H8.2C12) reaches a maximum in the mitochondria (Fig. 1B), whereas the holocytochrome *c* level in the mitochondria remains about the same as at 30 min (Fig. 3A), suggesting that at this time point either significant cytochrome *c* release is taking place or mitochondria are no longer able to convert apocytochrome *c* to holocytochrome *c*. The high level of holocytochrome *c* in the cytosol at 1 h (Fig. 3A) supports the former possibility. By 2 h, 7H8.2C12 still detects a high level of cytochrome *c* in the mitochondria (Fig. 1B), but the mitochondrial holocytochrome *c* is nearly completely released into the cytosol (Fig. 3A; also see Fig. 4D), suggesting that mitochondria can no longer convert the newly synthesized and transported apocytochrome *c* to holocytochrome *c* at this time point. By 4 h, 7H8.2C12 still detects a significant amount of cytochrome *c* in both mitochondria and cytosol (Fig. 1B), but the holocytochrome *c* in both compartments is barely detectable (Fig. 3A), suggesting that most of the cytochrome *c* detected by 7H8.2C12 at this time point is apocytochrome *c*. The nearly complete disappearance of holocytochrome *c* in both mitochondria and cytosol probably results from either its preferential release outside of the cells (54) or preferential degradation.

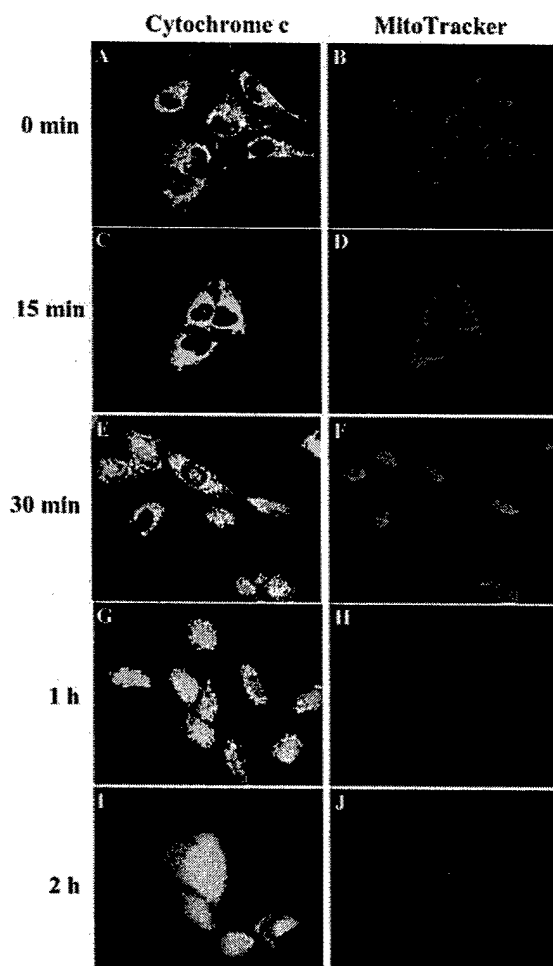
Next, we correlated cytochrome *c* alterations with the activation of the initiator caspase, caspase-9, and the executioner caspase, caspase-3. As shown in Fig. 3, B and C, caspase-9 (i.e. LEHDase activity) and caspase-3 (i.e. DEVDase activity) activation was observed at 1 h and reached the maximum levels by

2 h. In all apoptotic systems examined (Fig. 1 and data not shown), caspase activation (as judged by procaspase cleavage and/or substrate cleavage) was observed concomitant with or downstream of cytochrome *c* release.

Together, these results (Fig. 1, Fig. 2, A and B, and Fig. 3, A–C) suggest early cytochrome *c* up-regulation and translocation to the mitochondria preceding holocytochrome *c* release and caspase activation in BMD188-induced apoptosis of GM701 fibroblasts. Similarly, VP16-treated MDA-MB231 cells showed up-regulated cytochrome *c* mRNA as early as 30 min (Fig. 2B) and maximally induced cytochrome *c* protein, most of which accumulated in the mitochondria by 12 h (Fig. 1C). However, cytochrome *c* release from the mitochondria (not shown), caspase activation, and cell death (Fig. 1C) were not observed until ~24 h, again suggesting early cytochrome *c* up-regulation and translocation to the mitochondria preceding cell death. Similar sequence of alterations was also observed in serum-starved LNCaP cells.<sup>2</sup>

**Cytochrome *c* Release Involves Dynamic Changes in Bcl-2 Family Proteins, Loss of  $\Delta\psi_m$  and Opening of PTP**—Next, we carried out four sets of experiments in BMD188-treated GM701 cells to investigate the potential mechanisms of cytochrome *c* release. In the first, we examined the involvement of Bcl-2 family proteins as they play an essential role in regulating mitochondrial integrity and cytochrome *c* release (26, 55). Specifically, we examined three BH3-only proteins (i.e. Bim, Bad, and Bid), two multidomain proapoptotic proteins (i.e. Bax and Bak), and two multidomain anti-apoptotic proteins (i.e. Bcl-2 and Bcl-xL). As shown in Fig. 3D, dynamic changes were observed with these proteins. Bim, detected only in the mitochondria, was up-regulated (Fig. 3D), resulting from transcriptional

<sup>2</sup> J.-W. Liu, D. Chandra, and D. G. Tang, manuscript in preparation.



**FIG. 4. Loss of  $\Delta\psi_m$  precedes cytochrome *c* release during BMD188-induced GM701 cell apoptosis.** Shown are representative microphotographs of cytochrome *c* (A, C, E, G, and I) and MitoTracker labeling of mitochondria (B, D, F, H, and J) in GM701 cells treated with BMD188 (40  $\mu$ M) for 0 (A and B), 15 (C and D), and 30 min (E and F), and 1 (G and H), and 2 h (I and J). Another set of experiments with identical time points was performed to identify apoptotic cells using annexin V (see Supplemental Material Fig. 2S). See text for detailed discussions. Consistent with the Western blotting data and our previous observations (37), increased cytochrome *c* labeling was observed between 15 min and 1 h under the microscope, which was not well reproduced on micrographs. Original magnifications.  $\times 200$ .

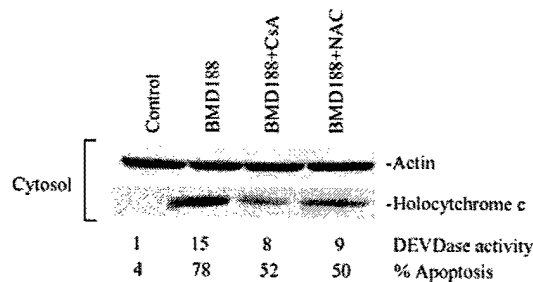
activation (not shown). Bad, detected mainly in the cytosol, showed a time-dependent translocation to the mitochondria until 2 h, when the mitochondria-associated Bad could hardly be detected. By 4 h, the mitochondria-associated Bad was undetectable, and the cytosolic Bad was mostly gone (Fig. 3D). Bid cleavage and activation, evidenced by the appearance of tBid in the mitochondria, was observed at 2 h and completed at 4 h (Fig. 3D), suggesting that this is a relatively late event, consistent with caspase-8 cleavage and activation starting from 2 h (not shown). Bax, detected equally in cytosol and mitochondria, showed somewhat complex alterations. Between 30 min and 2 h, Bax in both compartments was decreased. By 4 h, however, the cytosolic Bax was significantly decreased with a concomitant increase in the mitochondria (Fig. 3D). Bak, detected only in the mitochondria, on the other hand, showed a time-dependent increase that plateaued at 1 h (Fig. 3D). Interestingly, antibodies recognizing conformationally active Bax and Bak (Table I) revealed essentially identical changes in the two proteins (not shown). Bcl-2 and Bcl-x<sub>L</sub> showed very similar changes; both proteins showed a time-dependent increase exclusively in the mitochondria (Fig. 3D). The early increase in

the mitochondrial Bcl-x<sub>L</sub> may also involve its translocation from the cytosol as a decreased cytosolic Bcl-x<sub>L</sub> protein level was observed at 30 min post-treatment (Fig. 3D). These data suggest that dynamic changes in the Bcl-2 family proteins may be involved in cytochrome *c* release in BMD188-induced GM701 cell death.

In the second set of experiments, we examined the release of two other mitochondrial proteins that are significantly larger than cytochrome *c*: Smac, an ~25-kDa intermembrane protein (17, 18), and HSP60, a 60-kDa heat-shock protein localized in the matrix (56). As shown in Fig. 3E, Smac release, similar to holocytochrome *c* release (Fig. 3A), was observed 30 min after BMD188 treatment and plateaued by 2 h. Also similar to cytochrome *c*, the mitochondrial Smac protein level demonstrated a steady increase (Fig. 3E), suggesting an up-regulated transcription and/or translation. Interestingly, significant amounts of Smac still existed at 2 and 4 h when the majority of holocytochrome *c* had been released into the cytosol (compare Fig. 3, E and A), perhaps reflecting continuously up-regulated Smac synthesis just as significant amounts of cytochrome *c* in the mitochondria detected by 7H8.2C12 at these time points (see Fig. 1B). In contrast to holocytochrome *c* and Smac, HSP60 was maximally released as early as 30 min, and its levels in the mitochondria and cytosol remained relatively constant after 30 min until 4 h (Fig. 3E). Together, these data suggest that proteins significantly bigger than cytochrome *c* are also released during BMD188-induced apoptosis of GM701 cells.

In the third set of experiments, we used immunofluorescence microscopy and 6H2.B4, an antibody that recognizes only holocytochrome *c* in immunostaining (Table I), to examine the relationship between  $\Delta\psi_m$  and mitochondrial cytochrome *c* release. As shown in Fig. 4A, holocytochrome *c* in untreated GM701 fibroblasts was distributed in the mitochondria, which colocalized with MitoTracker labeling (Fig. 4B). No significant changes were observed in the staining patterns of cytochrome *c* and mitochondria by 15 min (Fig. 4, C and D). By 30 min, most cytochrome *c* was clearly localized in the mitochondria (Fig. 4E), consistent with only very low levels of holocytochrome *c* release detected on Western blotting (see Fig. 3A). However, most mitochondria had lost the  $\Delta\psi_m$  as indicated by the loss of MitoTracker labeling (Fig. 4F). Interestingly, nuclei were labeled by the MitoTracker dye starting from 30 min (compare Fig. 3F with B and D). Labeling with AlexaFluor-annexin V revealed similar degrees of low basal level apoptosis before and at 30 min (see Supplemental Material Fig. 2S, A–F). Consistent with Western blotting data (Fig. 3A), cytochrome *c* release became prominent by 1 h (Fig. 4G), when all mitochondria had lost the  $\Delta\psi_m$  (Fig. 4H). Meanwhile, apoptosis significantly increased (Supplemental Material Fig. 2S, G–H). By 2 h, cytochrome *c* was completely released from mitochondria (Fig. 4I), fully consistent with the Western blotting data (Fig. 3A). MitoTracker labeling was barely detectable (Fig. 4J), and apoptosis became widespread (Supplemental Material Fig. 2S, I and J). By 4 h neither cytochrome *c* nor MitoTracker labeling could be seen (not shown). These fluorescence microscopy results provide direct confirmation of the Western blotting data and also indicate that loss of  $\Delta\psi_m$  precedes holocytochrome *c* release from mitochondria. Similar changes in  $\Delta\psi_m$  and holocytochrome *c* release were also observed in MDA-MB-231 cells treated with VP16 (not shown).

Loss of  $\Delta\psi_m$  may result from opening of PTP, which in turn may be involved in cytochrome *c* release (10–12). To test this possibility, in the final set of experiments, we pretreated GM701 cells with cyclosporin A (CsA), which directly inhibits PTP (10–12), and NAC which indirectly inhibits PTP by blocking ROS generation (11, 12, 34), before apoptotic stimulation



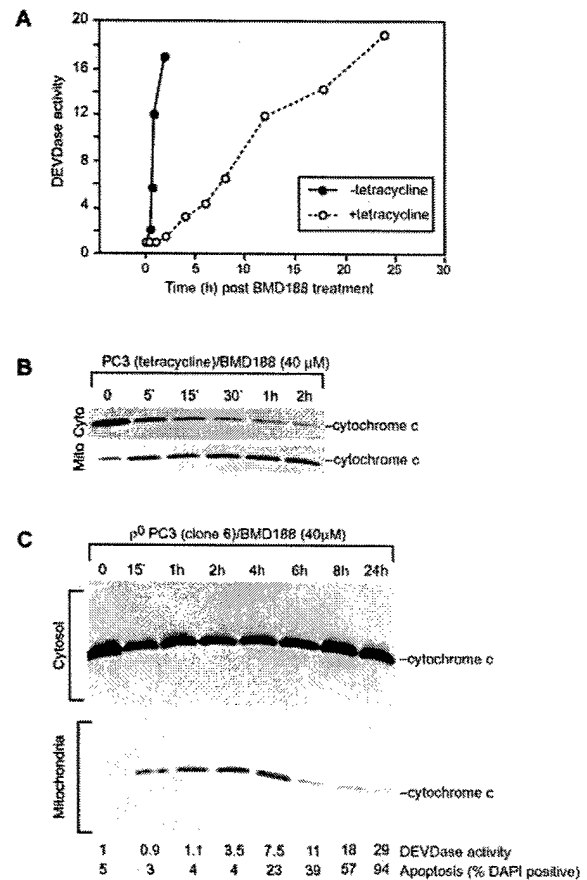
**FIG. 5. Inhibitors of PTP and ROS generation inhibits cytochrome c release, caspase activation, and apoptosis in GM701 cells treated with BMD188.** Cells were pretreated with CsA (5  $\mu$ M) or NAC (1  $\mu$ M) for 1 h, followed by addition of BMD188 (40  $\mu$ M) for 2 h. Cytosolic fractions were prepared as described under "Materials and Methods," and 40  $\mu$ g of proteins was used in Western blotting for holocytochrome c together with actin. The percentage of apoptotic cells was determined under a fluorescent microscope upon DAPI staining. DEVDase activity was measured using 40  $\mu$ g of whole cell lysates. Data shown are representative of at least three independent experiments.

with BMD188. As shown in Fig. 5, both CsA and NAC partially inhibited holocytochrome c release, caspase activation, and apoptosis in GM701 cells.

**Increased Transport of Cytosolic Cytochrome c into the Mitochondria Is Independent of MRC**—The preceding experiments demonstrate that apoptosis induced by multiple stimuli is preceded by an up-regulation of cytochrome c synthesis and transport into the mitochondria. Normal cytochrome c transport into the mitochondria has been shown to be mediated by a unique mechanism that does not depend on mitochondrial respiration or  $\Delta\psi_m$  (1, 53). To determine whether or not the continuously enhanced cytochrome c transport into the mitochondria during apoptosis depends on MRC or  $\Delta\psi_m$ , we employed two experimental strategies. In the first, we pretreated PC3 cells with tetracycline, which inhibits the mitochondrial protein synthesis and the MRC activity (1), prior to BMD188 treatment. As shown in Fig. 6A, inhibition of MRC function by tetracycline significantly delayed caspase activation, consistent with the previous observations (37) that BMD188-induced PC3 cell apoptosis requires MRC. Treatment with tetracycline also resulted in, as expected, the retention of most cytochrome c in the cytosol (Fig. 6B, 0 lane), because the protein is not needed for the electron transport (1). Upon BMD188 treatment, a time-dependent translocation of cytosolic cytochrome c to mitochondria was still observed (Fig. 6B), suggesting that the BMD188-induced increase of cytochrome c transport to the mitochondria does not depend on MRC *per se*.

In the second strategy, we treated MRC-deficient  $\rho^0$  PC3 cells (clone 6) (37) with BMD188. As in tetracycline-pretreated PC3 cells, the majority of cytochrome c in untreated  $\rho^0$  PC3 cells was retained in the cytosol (Fig. 6C, 0 lane). BMD188 treatment again induced a rapid translocation of cytosolic cytochrome c to the mitochondria (Fig. 6C). The accumulation of cytochrome c in the mitochondria reached peak level at ~4 h after BMD188 treatment, at which time increased caspase activation and apoptosis were observed (Fig. 6C). In wild-type, respiration-competent PC3 cells, 40  $\mu$ M BMD188 induced maximum caspase activation and killed ~90% of the cells within 4 h (37). In contrast, BMD188 at the same dose induced similar levels of caspase activation and apoptosis in  $\rho^0$  PC3 cells only after ~24 h treatment (Fig. 6C). Together with the tetracycline experiments, these observations suggest that the BMD188-induced cell death but not cytochrome c translocation to the mitochondria depends on the MRC function.

**Up-regulation of Cytochrome c Alone Is Insufficient to Induce Apoptosis but Potentiates Cell Death by BMD188**—To test whether increased cytochrome c synthesis and enrichment in



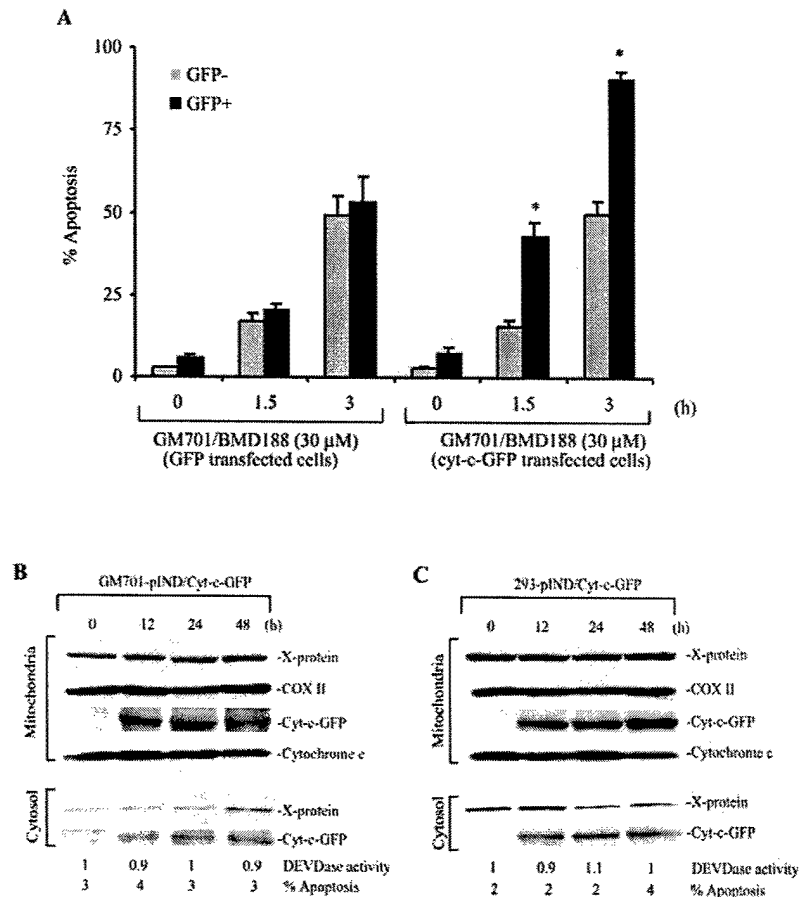
**FIG. 6. BMD188-induced PC3 cell apoptosis, but not BMD188-stimulated cytochrome c translocation to the mitochondria, depends on MRC.** A, tetracycline inhibits BMD188-induced caspase activation in PC3 cells. PC3 cells were pretreated with tetracycline (1  $\mu$ g/ml) for 1 h and then treated with BMD188 (40  $\mu$ M) in the presence of tetracycline. Whole cell lysates were prepared at the time points indicated and used to measure DEVDase activity. B, MRC-inhibited PC3 cells treated with tetracycline still show a time-dependent translocation of cytochrome c from cytosol to the mitochondria upon BMD188 treatment. Thirty  $\mu$ g/lane of the fractionated cytosolic or mitochondrial proteins (see "Materials and Methods") was used in Western blotting for cytochrome c. C, respiration-deficient  $\rho^0$  PC3 (clone 6) cells (37) still show cytochrome c translocation to the mitochondria in response to BMD188 treatment. Thirty  $\mu$ g/lane of the fractionated cytosolic or mitochondrial proteins was used in Western blotting for cytochrome c. In the meantime,  $\rho^0$  PC3 cells treated with BMD188 for the same time intervals were also used to measure DEVDase activity and apoptosis.

the mitochondria contribute to apoptosis induction, we transiently transfected GM701 cells with an expression plasmid encoding cytochrome c-GFP (cyt-c-GFP) fusion protein, which has been shown previously (51) to localize effectively to the mitochondria and to be released from the mitochondria during staurosporine-induced apoptosis. Twenty-four h after transfection, cells were treated with BMD188 for various lengths of time, followed by quantification of apoptotic nuclei in both GFP-positive as well as GFP-negative cells. As shown in Fig. 7A, GM701 cells transfected with the GFP alone (control vector) showed similar levels of apoptosis in both GFP<sup>+</sup> and GFP<sup>-</sup> populations. In contrast, in GM701 cells transfected with the cyt-c-GFP, the BMD188-induced apoptosis was significantly enhanced (Fig. 7A). For example, 3 h after BMD188 treatment, >90% of the GFP<sup>+</sup> cells was apoptotic compared with ~60% of apoptosis in GFP<sup>-</sup> cells (Fig. 7A).

The above results suggest that up-regulation of cytochrome c by enforced expression potentiated apoptosis induced by BMD188. To address whether up-regulation of cytochrome c is by itself sufficient to induce apoptosis, we used an ecdysone-



FIG. 7. Overexpression of cytochrome *c* enhanced BMD188 induced apoptosis (A) but cytochrome *c* overexpression by itself (i.e. without stimulation) is insufficient to trigger apoptosis (B). A, GM701 fibroblast cells were transfected with plasmids encoding GFP or cyt-*c*-GFP. Twenty four h later, cells were treated with BMD188 (30  $\mu$ M) for the times indicated. Cells were labeled live with DAPI 15 min before the end of the treatment followed by fixation in 4% paraformaldehyde. Apoptosis was quantified by nuclear morphology. The results are presented as % of apoptosis in both GFP<sup>+</sup> or GFP<sup>-</sup> cell populations. Values represent the mean  $\pm$  S.D. from three separate experiments. \*, significantly different ( $p < 0.01$ , Student's *t* test) compared with GFP<sup>-</sup> cells. B and C, Western blot analysis of cytosolic and mitochondrial fractions from GM701-pIND/cyt-*c*-GFP (B) and 293-pIND/cyt-*c*-GFP (C) cells induced by ponasterone (2  $\mu$ M) for the time intervals indicated. Twenty five  $\mu$ g/lane of proteins from each sample was separated by 15% SDS-PAGE. Following protein transfer, membranes were probed and reprobed with antibodies against COX II, cytochrome *c* (which recognizes only endogenous cytochrome *c* but not cyt-*c*-GFP), or GFP (which recognizes GFP or cyt-*c*-GFP). Apoptosis and DEVDase activity were determined as described under "Materials and Methods." Data presented are representative of three separate experiments.



inducible system to establish transcriptionally inducible cytochrome *c* in GM701 and 293 cells. Double stable clones of each cell type expressing inducible GFP or cyt-*c*-GFP in response to a ligand such as ponasterone were generated in two steps as detailed under "Materials and Methods." The resultant cells, i.e. GM701-pIND/GFP, GM701-pIND/cyt-*c*-GFP, 293-pIND/GFP, and 293-pIND/cyt-*c*-GFP cells, were treated with 2  $\mu$ M ponasterone for various time intervals to induce the expression of GFP or cyt-*c*-GFP. As shown in Fig. 7, B and C, ponasterone induced a rapid induction of cyt-*c*-GFP, the majority of which, like endogenous cytochrome *c*, was localized in the mitochondria, whereas ponasterone-induced GFP alone was mainly localized in the cytosol (not shown). Ponasterone treatment did not affect the expression of endogenous cytochrome *c* or COX II (Fig. 7, B and C). Up-regulation of cyt-*c*-GFP in both GM701 (Fig. 7B) and 293 (Fig. 7C) cells did not lead to increased caspase activation or apoptosis.

In the above experiments, we utilized the cyt-*c*-GFP fusion protein, which, although being able to correctly target to the mitochondria (see Ref. 51; data not shown), might not be fully functional as an electron carrier in the MRC because of the presence of the GFP tag. To exclude this possibility, we made a new expression construct, pCMS-EGFP/cyt-*c*, in which the human cytochrome *c* (without any tag) and the EGFP are independently synthesized from two separate promoters (see "Materials and Methods"). In this way, cytochrome *c* and GFP are made as two separate proteins. By using this vector, we carried out experiments similar to those in Fig. 7, B and C. As shown in Supplemental Material Fig. 3S, as early as 24 h after transient transfection of pCMS-EGFP/cyt-*c*, the cytochrome *c* level was significantly up-regulated (~4-fold) only in the mitochondria, compared with untransfected cells or cells transfected with the empty vector. As expected, GFP was detected

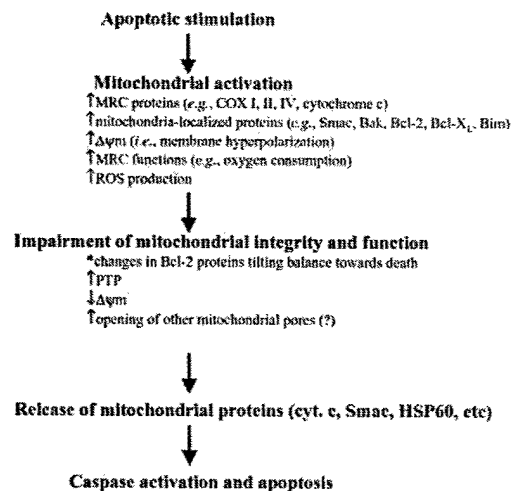


FIG. 8. A scheme illustrating the sequence of events in MADAP, highlighting the potentially critical role of early mitochondrial activation in causing subsequent mitochondrial dysfunction and cytochrome *c* release.

only in the cytosol of the transfected cells (Fig. 3S). Because the up-regulated cytochrome *c* was detected only in the mitochondria, it is reasonable to think that this exogenous cytochrome *c*, just like the endogenous protein, should be functional in participating in MRC electron relays. Nevertheless, there was no significantly increased cell death at 24 (Fig. 3S) to 72 h (not shown) after the transfection. Together, this set of experiments provides independent supporting evidence that up-regulation of cytochrome *c* alone is insufficient in inducing apoptosis.

## DISCUSSION

**Up-regulation of MRC Proteins and Mitochondrial Activation Early during Apoptosis Induction**—Our previous studies (37) and the present work show that apoptosis induced by multiple stimuli is temporally preceded by an early induction of MRC proteins such as cytochrome *c* and COX II. For example, induction of cytochrome *c* and COX II mRNAs and proteins in GM701 cells treated with BMD188 occurs within 10 min (Fig. 1B and 2A), preceding caspase activation and death, which becomes detectable at 30 min to 1 h (Figs. 1B and 3; Supplemental Material Fig. 2S). Similarly, up-regulated cytochrome *c* and COX II mRNAs and proteins in MDA-MB231 cells treated with VP16 occur much earlier than caspase activation and apoptosis (Figs. 1C and 2). Recent work by others has also revealed increased expressions of cytochrome *c* and COX II preceding cell death in Jurkat cells treated with camptothecin (57) or breast cancer cells treated with teniposide (58). The increased cytochrome *c* and COX II proteins in these apoptotic model systems appear to result from the transcriptional activation of the respective genes, as illustrated in BMD188-treated GM701 cells (Fig. 2A) and VP16-treated MDA-MB231 cells (Fig. 2B). Because COX II is encoded by the mitochondrial genome and cytochrome *c* the nuclear genome, these observations suggest that the MRC components encoded by both genomes are coordinately induced early in apoptotic signal transduction by these stimuli. In support, two other MRC proteins, COX I and COX IV, also encoded by the mitochondrial and nuclear genomes, respectively (1), are similarly up-regulated during apoptosis induced by BMD188 (37), camptothecin (57), and teniposide (58).

Why should cells up-regulate these MRC proteins in response to apoptotic stimuli of diverse mechanisms of action (see Refs. 37, 57, and 58; this study)? Multiple pieces of evidence suggest that the up-regulation of MRC proteins may represent one aspect of a more global mitochondrial activation response (Fig. 8). First, not only MRC proteins but also many mitochondrially localized, non-MRC proteins are up-regulated. For example, in BMD188-induced GM701 cell death, Bcl-2 family proteins Bim, Bak, and Bcl-2 as well as Smac, all encoded by the nuclear genes and normally localized exclusively in the mitochondria, are up-regulated (Fig. 3, D and E). Similarly, both Bim and Bcl-2 are also induced in serum-starved LNCaP cells and VP16-treated MDA-MB231 cells.<sup>2</sup> Second, apoptosis induced by many of these apoptotic stimuli exemplified by BMD188 (37), camptothecin (57), staurosporine (13), Fas (59), and Mn(II) (38) is preceded by an early hyperpolarization of the  $\Delta\psi_m$  (Fig. 8), thus indicative of enhanced electron transport and MRC activity. Third, these inducers rapidly up-regulate the oxygen consumption capacity of the cells (e.g. 37, 57, 58) or COX activity (35), suggesting that the up-regulated MRC components are functionally participating in the electron transport. Finally, mitochondria represent the primary site of ROS generation in the cells, and enhanced MRC activity is generally accompanied by increased ROS production (5–7). Indeed, increased ROS generation has been observed early in apoptosis induced by most of these stimuli including TNF- $\alpha$ , Fas, BMD188, nordihydroguaiaretic acid, camptothecin, Fas, Mn(II), retinoid CD437, ceramide, short chain fatty acids, hypoxia, and deprivation (31–43, 57–59). Altogether, these observations suggest that one of the common events in apoptosis induced by a wide spectrum of stimuli is early mitochondrial activation manifested as increased synthesis of mitochondrially localized (both MRC and non-MRC) proteins and increased  $\Delta\psi_m$ , oxygen consumption, and ROS production (Fig. 8).

This early mitochondrial activation could represent a defensive response of cells to various stresses. However, the fact that

cells lacking a functional MRC (i.e.  $\rho^0$  cells) or cells with deficient MRC function due to either chemical blocking or genetic mutation/deletion of individual respiratory proteins are often resistant to apoptosis induction (31–43) (Supplemental Material Fig. 1S; see discussion in Introduction) strongly suggests that the MRC and early mitochondrial activation are also causally involved in apoptosis induction (see below). In this sense, apoptosis triggered by the stimuli that cause early mitochondrial activation and require the mitochondrial activation for the apoptotic effect can be called mitochondrial activation-dependent apoptotic pathway or MADAP.

**Importance of Early Mitochondrial Activation and Cytochrome *c* Up-regulation in MADAP**—How might early mitochondrial activation contribute to apoptosis induction? We shall address this question later in the context of cytochrome *c* release. However, a detailed look at the potential role of cytochrome *c* movement in BMD188-induced GM701 cell death may shed some light on this question. Early upon stimulation the apoptotic signals rapidly up-regulate cytochrome *c*, some of which translocates to the mitochondria. As the increased cytochrome *c* synthesis in the cytosol and transport into the mitochondria continue and intensify, holocytochrome *c* is gradually released from the mitochondria into the cytosol to initiate apoptosis formation. Remarkably, increased cytochrome *c* synthesis and transport are still observed even when holocytochrome *c* is nearly completely released from the mitochondria (Figs. 1B, 2A, and 3A). Therefore, cytochrome *c* undergoes cyclic changes in this apoptotic model, i.e. increased apocytochrome *c* synthesis in the cytosol  $\rightarrow$  increased apocytochrome *c* transport into the mitochondria  $\rightarrow$  increased holocytochrome *c* accumulation in the mitochondria  $\rightarrow$  increased holocytochrome *c* release into the cytosol  $\rightarrow$  the whole cycle continues until all holocytochrome *c* is released from the mitochondria and until mitochondria no longer have the ability to convert up-regulated apocytochrome *c* to holocytochrome *c*.

Several pieces of evidence suggest that the increased cytochrome *c* translocation to and its accumulation in the mitochondria might contribute to apoptosis induction. First, in all the cases studied, the translocation of cytochrome *c* to and its accumulation in the mitochondria occur prior to caspase activation and cell death (Fig. 1, A–E and data not shown). Second, in  $\rho^0$  and tetracycline-treated PC3 cells, although cytochrome *c* translocation still occurs, its peak accumulation in the mitochondria is delayed, i.e. from ~1 to 2–4 h (37) (Fig. 6, B and C of this work). In the meantime, caspase activation as well as cell death are also delayed (Fig. 6; 37). Third, inhibition of *de novo* mRNA synthesis by A/D or protein synthesis by CHX inhibits BMD188 and VP16-induced up-regulation in COX II and cytochrome *c* as well as apoptosis (Fig. 2). Although A/D and CHX may likely affect many other gene and protein targets, it is reasonable to think that their inhibitory effects on cytochrome *c* up-regulation, at least partially, contribute to their inhibition of apoptosis. Finally, enforced overexpression of exogenous cytochrome *c*, which also rapidly translocates to the mitochondria (not shown), significantly potentiates apoptosis (Fig. 7A). Interestingly, simply up-regulating cytochrome *c* expression is insufficient to trigger apoptosis; in the absence of an apoptotic inducer, up-regulated cytochrome *c* does not increase spontaneous cell death (Fig. 7, B and C; Supplemental Material Fig. 3S). These observations suggest that the accumulation of cytochrome *c* in the mitochondria may represent only one of the apoptosis-initiating factors and that it is the combination of many factors during mitochondrial activation that eventually leads to mitochondrial dysfunction, cytochrome *c* release, and caspase activation (see below).

How is the newly synthesized cytochrome *c* transported into

the mitochondria during MADAP? Normally, most cytochrome *c*, upon synthesis, is immediately transported to the mitochondria in which the protein turns into holocytochrome *c* as the result of binding to the heme group, and this transport process utilizes a unique import pathway that does not depend on the MRC or  $\Delta\psi_m$  (1, 53). Likewise, the increased transport of cytochrome *c* to the mitochondria in BMD188-induced PC3 cell death does not depend on the MRC or  $\Delta\psi_m$ , as it also occurs in  $\rho^0$  cells or when MRC is inhibited by tetracycline (Fig. 6). In support of this conclusion, increased cytochrome *c* accumulation in the mitochondria is still observed long after the mitochondria have lost the  $\Delta\psi_m$  (compare Figs. 1B, 3A, and 4).

Our observation that cytochrome *c* can be transcriptionally up-regulated leading to increased apocytochrome *c* proteins in the cytosol has a practical implication. In the literature, frequently only the increased cytosolic cytochrome *c* levels are shown, and this is used as evidence of cytochrome *c* release from the mitochondria. Without using an antibody that specifically recognizes the holocytochrome *c* (Table I) and without demonstrating correspondingly decreased mitochondrial cytochrome *c*, however, it will be unable to distinguish whether the increased cytochrome *c* in the cytosol results from mitochondrial release or from the transcriptional up-regulation of the gene.

**How Might Cytochrome *c* Be Released during MADAP?**—The apoptogenic holocytochrome *c* is normally sequestered in the mitochondrial intermembrane space. The outer mitochondrial membrane (OMM) has a limited permeability allowing the passage of molecules <1.5 kDa, and the inner mitochondrial membrane (IMM) is essentially impermeable. Although still highly debatable, three major models have been proposed to explain how cytochrome *c* might be released from the mitochondria during apoptosis (60). In the first, proapoptotic Bcl-2 family proteins, Bax and Bak in particular, directly form pores on OMM to release selectively cytochrome *c* without major effects on mitochondrial function (61–63). In the second model, apoptotic signals open the PTP resulting in the loss of  $\Delta\psi_m$  and swelling of the mitochondrial matrix, which causes eventual OMM rupture, nonselective OMM permeabilization, and cytochrome *c* release (63–65). In the third model, apoptotic signaling evokes the opening of a voltage-independent megapore termed MAC (mitochondria apoptosis-induced channel) (66). MAC is distinct from PTP in that it does not have voltage-dependent anion channel (VDAC, located in OMM) as a component, and it displays multiple conductance levels, with a peak single channel opening of ~2.5 nS, corresponding to a pore diameter of ~4.5 nm (66). Therefore, MAC is significantly bigger than the Bax/Bak channel or PTP (66).

None of these models alone seems to be able to explain completely how cytochrome *c* might be released during MADAP. Instead, dynamic changes in Bcl-2 family proteins, opening of PTP and loss of  $\Delta\psi_m$ , and opening of much larger pores (that can allow the release of HSP60 from the matrix) all seem to be involved. For example, in BMD188-induced GM701 cell death, all three BH3 domain-only proteins, *i.e.* Bim, Bad, and Bid, are activated; Bim is up-regulated transcriptionally (Fig. 3D and data not shown); Bad rapidly translocates to the mitochondria, and Bid is cleaved late during apoptosis (Fig. 3D). In contrast, the multidomain Bcl-2 proteins show complex alterations; both Bcl-x<sub>L</sub> and Bcl-2 are induced and concentrated in the mitochondria, and Bak is induced whereas Bax is reduced early during apoptosis induction (Fig. 3D). Similar alterations such as rapid induction of Bim mRNA and protein have also been observed in MDA-MB231 cells treated with VP16 and LNCaP cells subjected to serum starvation.<sup>2</sup> Because the Bcl-2 proteins normally function in the mitochondria to maintain the

organelle integrity and functional homeostasis (26, 55, 63, 64, 67), these dynamic changes may reflect the life-and-death “battle” among these proteins. Thus, it is possible that, as Bim and Bad are activated and Bak is induced, Bax is down-regulated, and prosurvival Bcl-2 and Bcl-x<sub>L</sub> are up-regulated in order to prolong the cell survival. As apoptotic stimulation continues, more Bim and Bak are induced, and more Bad is translocated to the mitochondria, tilting the balance toward cell death. In this scenario, cytochrome *c* might be released through the Bax/Bak channel (as a significant amount of Bax is always present in the mitochondria) or through Bak alone, which has been shown recently (68) to play a critical role in mediating cytochrome *c* release in anticancer drug-induced apoptosis.

The Bax/Bak pores are small (0.5 nS; 66) and are thought to release selectively cytochrome *c* without significantly affecting mitochondrial parameters such as membrane permeability and matrix volume (61–63). In apoptosis induced by BMD188 (37) and many other stimuli (*e.g.* see Refs. 13, 38, 57, and 59), there is an early IMM hyperpolarization and increased  $\Delta\psi_m$  followed by subsequent loss of  $\Delta\psi_m$ . Furthermore, at least in the case of BMD188-induced GM701 cell death, proteins much larger than cytochrome *c* (*i.e.* 25-kDa Smac and 60-kDa HSP60) are also released from mitochondria. Together, these observations suggest that cytochrome *c* release in these apoptotic systems may involve opening of PTP or MAC or some other pores in addition to Bcl-2 family proteins (Fig. 8). The supporting evidence comes from the loss of the  $\Delta\psi_m$  at 30 min when the majority of cytochrome *c* in most cells is still in the organelle (Fig. 4), suggesting PTP opening prior to cytochrome *c* release. More importantly, BMD188-induced cytochrome *c* release and subsequent caspase activation and cell death in GM701 cells can be inhibited by CsA, which inhibits cyclophilin D, an important component of the PTP, as well as by NAC, which indirectly inhibits PTP (11, 12). The inhibitory effect of NAC also suggests ROS production by BMD188, as observed previously (37). It is interesting to note that the patterns of cytochrome *c* release and Smac are very similar (Fig. 3, A and D), suggesting that these two intermembrane proteins may utilize the same (or similar) channel or pore for their exodus. Surprisingly and intriguingly, the matrix protein HSP60 is maximally released into the cytosol much earlier, at a time when cytochrome *c*/Smac release has just started (see Fig. 3, A and D). These differential release kinetics suggest the following: 1) the release of these individual proteins is specific, which cannot be accounted for by nonspecific rupture of OMM; and 2) different channels or pores are probably utilized to release different proteins.

It is noteworthy that Bim is rapidly and commonly induced in the three MADAP systems we examined in detail, *i.e.* BMD188-treated GM701 cells (this study), serum-deprived LNCaP cells,<sup>2</sup> and VP16-treated MDA-MB231 cells,<sup>3</sup> suggesting that this may be the key BH3-only molecule in initiating MADAP. Interestingly, Bim has been shown recently to induce both Bax/Bak-dependent and Bax/Bak-independent cytochrome *c* release (69). In the latter mechanism, Bim directly interacts with VDAC and triggers VDAC-dependent cytochrome *c* release (69). Because VDAC is an integral component of PTP and also forms pores with Bax (63–65), which in turn seems to be part of the MAC (66), it is possible that all these proteins together form very dynamic pores/channels of different sizes and selectivity at the contact sites of IMM and OMM, which are opened by BH3-only proteins such as Bim and closed by anti-apoptotic proteins such as Bcl-2 and Bcl-x<sub>L</sub>.

In summary, our data presented herein, together with other

<sup>2</sup> D. Chandra, J.-W. Liu, and D. G. Tang, unpublished observations.

data (31–45, 57, 58) suggest the apoptotic model presented in Fig. 8. In response to a wide diversity of apoptotic stimuli, cells immediately wage a defensive response characterized by mitochondrial activation, manifested by rapid up-regulations of multiple MRC proteins and enhanced MRC activities such as oxygen consumption. In the meantime, Bcl-2 proteins undergo dynamic alterations in attempt to keep the cells alive. In the persistent apoptotic stimulation, the increasing ROS production as a result of continuously increased MRC activation and cytochrome *c* accumulation in the mitochondria results in the opening of PTP and/or other pores and loss of  $\Delta\psi_m$ , which, together with more pro-apoptotic changes in the Bcl-2 family proteins, leads to the release of holocytochrome *c* and, subsequently, activation of caspases.

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